Molecular Quantification of Response to Therapy and Remission Status in TEL-AML1-Positive Childhood ALL by Real-Time Reverse Transcription Polymerase Chain Reaction


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INTRODUCTION

Although TEL-AML1 positivity [translocation t(12;21)(p13;q22)], detected in 20–25% of initial childhood acute lymphoblastic leukemia (ALL), has been associated with an excellent prognosis, its positive predictive value is insufficient for appropriate treatment stratification considering reported prevalence in relapsed ALL (3–28%). Molecular quantification of response to therapy by PCR-based methods has been shown to improve risk assessment. Here, we report on the sensitive quantification of leukemia-specific TEL-AML1 fusion transcript levels normalized to β-actin expression (sensitivity thresholds, 10^{-5}) by a novel real-time reverse transcription-PCR (RQ-RT-PCR) based on fluorescent TaqMan technique providing early and rapid evidence on the treatment efficacy of children with initial or relapsed TEL-AML1 ALL enrolled in frontline or relapse trials of the Berlin-Frankfurt-Münster (BFM)-Study Group. In initial ALL, TEL-AML1 /β-actin decrease was ≥10-fold in 50% of patients after induction therapy (day 33) and stayed TEL-AML1-negative throughout therapy, which suggested high sensitivity of leukemic cells to antineoplastic therapy. The remaining patients were still TEL-AML1-positive before reintensification (ratios, 0.7 ± 0.2), and in relapsed ALL, TEL-AML1 /β-actin decrease was generally less pronounced at corresponding time points, and conversion to TEL-AML1 negativity was observed in 40% of patients. Most notably, subsequent relapses occurred only among molecular poor responders, whereas all early responders remain in their second complete remission. In conclusion, real-time quantification of TEL-AML1 /β-actin kinetics distinguishes distinct molecular response groups, and provides indications capable of directing therapeutic interventions for patients with TEL-AML1 ALL. Before considering modification of therapy, results should be interpreted cautiously taking into account the long duration of remission associated with TEL-AML1 ALL.

MATERIALS AND METHODS

Patients and Treatment

BM samples from 33 children with TEL-AML1 AND BCP-ALL were analyzed by RQ-RT-PCR at time point of ALL diagnosis (13 initial ALL, 20 relapses). Sequential BM samples from 12 children with initial TEL-AML1 AND ALL and 10 children with relapsed TEL-AML1 ALL were obtained at diagnosis and at regimen-defined diagnostic time points during therapy (34 and 22 follow-up samples, respectively). All of the patients were treated according to frontline trial ALL BFM 95 (24) or relapse trial ALL-REZ BFM 96 (25) of the BFM study group. The majority of analyses was performed prospectively. Written informed consent was obtained from the patients or guardians. The studies ALL BFM and ALL-REZ BFM were approved by the Institutional Review Boards of the Medical School Hannover and the Humboldt-University at Berlin, respectively.

Molecular Detection of the TEL-AML1 Fusion mRNA

Standard Preparation. The TEL-AML1 fusion transcript was amplified by RT-PCR from the BCP-ALL cell line REH known to carry the respective
translocation, was ligated into the pCR2.1 vector (TOPO TA Cloning kit; Invitrogen, Leek, the Netherlands), and was sequenced. Plasmids were digested with HindIII and XbaI (Boehringer Mannheim, Mannheim, Germany), extracted from 3% agarose gel, and reamplified. Finally, these fragments were purified (PCR Purification kit; Qiagen, Hilden, Germany) and measured in a photometer, and molecule concentrations were calculated. Sensitivity of the assay was assessed in serial dilutions ranging from 10^7 to 10^-7 molecules per 100 ng DNA [in herring sperm DNA and TE buffer (pH 8.0)].

**Samples.** BM samples were collected into sterile heparin-containing tubes, and mononuclear cells were obtained after centrifugation on a Ficoll-Hypaque gradient. All of the patient samples at diagnosis contained more than 94% leukemic cells. Total RNA isolation and reverse transcription into cDNA have been described elsewhere (19, 20, 26). The quality of RNA was visualized on an ethidium bromide-stained 1% agarose gel.

**RQ-RT-PCR.** Because of the occurrence of variant TEL-AML1 fusion transcripts (8, 20, 27, 28), the fluorogenic probe was designed to hybridize to the coding strand of TEL segment (5'-TCTCCCCGCTGAAAGCACA-3'; exon 5) consistently present in the fusion event. TaqMan PCR for TEL-AML1 was performed using primers corresponding to the exon 5) and to the AML1 part of the fusion (5'-gAgCaAgCgGACGACG-3'; exon 3), which enabled the detection of both variants.

The probe was labeled with 6-carboxy-fluorescein phosphoramidite (FAM) at the 5' end and as quencher 6-carboxy-x-rhodamine (TAMRA) (TIB Molbiol, Berlin, Germany), and was sequenced. Plasmids were differentiated by analysis of serial standard dilutions of TEL-AML1, and was generated by analysis of serial standard dilutions of TEL-AML1 DNA.

**RESULTS**

**Real-Time PCR for Detection of TEL-AML1 Fusion.** For quantification of cDNA by real-time fluorescence PCR, a calibration curve was generated by analysis of serial standard dilutions of TEL-AML1 and beta-actin. For this purpose, each calibrator was correlated with its threshold cycle (Ci), i.e., the cycle number when a given sample becomes positive, defined as a measured fluorescence >10 SD above the background fluorescence. The amplification curves of the calibrators and the calibration curve generated by the Sequence Detector computer software are shown in Figs. 1 and 2, respectively. Regarding sensitivity, the assay was able to detect 10 TEL-AML1 or 10 beta-actin copies per 100 ng of cDNA. Each measurement was performed in triplicate. The intra-assay CV was <5%, whereas the interassay CV was <10%, as calculated by the variation of the mean (29, 30).

Furthermore, although cDNA of the REH cell line and peripheral blood of donors exhibited 5.1 ± 2.1 × 10^6 beta-actin copies/100 ng cDNA, the respective DNA samples remained stably negative over 45 cycles of PCR (29). Pseudogenes and genomic DNA of beta-actin were not amplified, which demonstrated the specificity of this technique (29).

**Degradation Rates for TEL-AML1 and beta-actin Transcripts.** RQ-RT-PCR demonstrated that the stability and degradation rates of TEL-AML1 and beta-actin transcripts were similar (0.25 and 0.27 log after 24 h; 0.64 and 0.66 log after 48 h, respectively). The TEL-AML1: beta-actin copy ratio remained stable during the same time period.

**Clinical Samples at Diagnosis of Initial and Relapsed TEL-AML1+ ALL.** The quantity of TEL-AML1 was normalized to the absolute quantity of beta-actin transcript, and the result was expressed as the ratio of TEL-AML1:beta-actin copies. At presentation, the median level of TEL-AML1:beta-actin ratio in BM samples of 13 children with initial ALL was 0.21 (range, 0.34–0.12; see also Table 1) and did not differ from the ratio in 20 relapsed ALL (median, 0.19; range, 0.95–0.15; Table 2). The clinical characteristics are presented in Tables 1 and 2.

**Therapy Response and Follow-Up Examinations.** Children with initial TEL-AML1+ ALL were stratified to BFM standard or medium risk groups (31). All of the patients were good responders to prednisone (<1000 blasts/μl, day 8), and went into morphological CR after induction. The reduction of TEL-AML1 expression in BM samples from 12 patients with initial ALL was monitored during early induction (days 0, 15, and 33) and before reintensification (day 148) of protocol ALL BFM 95 (Fig. 3A). For serial analyses, the TEL-AML1: beta-actin ratios obtained at diagnoses were set to one, and the follow-up levels were calculated in relation to this ratio.

Serial RQ-RT-PCR analysis during induction disclosed three different molecular responder groups. Both the very rapid (three patients) and the rapid responders (three patients) reached CR, respectively, and BM samples remained negative throughout further therapy. The remaining 6 (50%) of 12 patients showed a slower response to therapy (TEL-AML1 reduction ≤ 4 logs), and were TEL-AML1+ before reintensification (TEL-AML1:beta-actin ratio, 10^-2 to 10^-4). There were no differences in clinical characteristics between patient groups. At a median observation time of 21 months (range, 12–56 months), all of the patients were in first CR (Table 1). The relapse trial, ALL-REZ BFM 96, consists of alternating
DISCUSSION

The TEL-AML1 fusion not only characterizes the most frequent genetic rearrangement in initial childhood ALL (20–25%) but its presence has also been associated with a favorable prognosis (9–11, 28, 32). In clinical studies on initial ALL, probability of event-free survival (EFS) at 4 years was as high as 90–100% for TEL-AML1+ patients (9, 10, 32). These results certainly do not represent final outcome considering that, regardless of the different prevalence of TEL-AML1 positivity at relapse of BCP-ALL (range, 3–28%), TEL-AML1+ leukemia is biologically characterized by a long duration of first CR and that the majority of relapses (80%) occur off-therapy (median, 46 months; range, 13–125 months; Refs. 18–23, 33). The prevalence of TEL-AML1 positivity in our ongoing prospective study on first relapse of BCP-ALL is ~17% (31 of 178 children;33).

Obviously, the predictive value of TEL-AML1 positivity alone is insufficient to stratify patients to appropriate treatment arms. This is of particular importance for the decision on high-risk BM transplantation procedures. In this study, we have shown that quantitative real-time PCR technique allows rapid and sensitive analysis of absolute TEL-AML1 template amounts without any post-PCR steps to assess response to therapy and to monitor the course of disease reliably. RQ-RT-PCR could clearly distinguish between early and late molecular responders in initial TEL-AML1+ ALL. One-half of these patients achieved molecular remission after induction therapy (day 33). In the other half, the decline of TEL-AML1:β-actin ratio was less pronounced (1 to 4 logs), and TEL-AML1 fusion transcripts were permanently detectable during therapy. The critical TEL-AML1:β-actin threshold in ALL after induction or before reintensification therapy associated with an increased risk of relapse needs to be defined in a larger study cohort. Moreover, correlation of RQ-RT-PCR analysis results with final outcome of patients with TEL-AML1+ ALL requires longer observation times because of the known long periods of remission associated with TEL-AML1 positivity.

In DNA-based MRD studies, patients with a low degree of MRD (≤10−4) at the end of induction have a lower risk of relapse than those with MRD levels ≥10−2 (1, 2). Moreover, ALL patients demonstrating an early molecular response to therapy (20% of all initial ALL; MRD, ≤10−4 on day 15) have a significantly better outcome than those with a higher MRD degree (4). Consistent with this report, 25% of initial TEL-AML1+ ALL were early responders.

In relapsed TEL-AML1+ ALL, although all of the patients were stratified to the same risk-group of the relapse trial ALL-REZ BFM (S2), real-time PCR could discriminate between early, late, and non-responders. The subset converting to molecular TEL-AML1 negativity courses of polychemotherapy given in ~3-week intervals (20, 25).

Sequential quantification of TEL-AML1 expression was performed at diagnosis and before courses 2–5 (day 21, 42, 63, and 84, respectively) in BM samples from 10 patients with a combined relapse of ALL (ALL-REZ BFM 96, strategy group S2; Ref. 20; Fig. 3B). The median of first remission duration was 43 months (range, 31–109 months). In comparison with patients with initial ALL, the reduction of TEL-AML1 transcripts was generally slower despite more intensive chemotherapy. Before the second course of polychemotherapy (day 21), one group (four patients) demonstrated a reduction of 3 to 4 logs and had ≤10−3 TEL-AML1 fusion transcripts before the third course (day 42). No relevant reduction of TEL-AML1 transcript values was detected in the other group (six patients) before the second and third courses of polychemotherapy (<1 log) and decreased only very gradually in most cases during further therapy. Most significantly, a median observation time of 21.5 months (range, 9–39 months), relapses have occurred only among the molecular poor responders. Three of six patients with a TEL-AML1:β-actin ratio higher than 10−3 before the second and third courses suffered a relapse at 9 months (patient 2), 23 months (patient 1), and 38 months (patient 15), in contrast to none among the rapid responders (19–34.5 months; Fig. 3 and Table 2).

Table 1 Characteristics of children with initial TEL-AML1-positive ALL, TEL-AML1:β-actin ratio at diagnosis, and treatment outcome

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (months)</th>
<th>ALL subtype</th>
<th>BM blasts (%)</th>
<th>TEL-AML1:β-actin</th>
<th>Frontline trial</th>
<th>Observation time (months)</th>
<th>Present clinical status</th>
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<tr>
<td>3</td>
<td>F</td>
<td>29</td>
<td>Common</td>
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<td>ALL BFM 95</td>
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<td>First CR</td>
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<td>4</td>
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<td>54</td>
<td>Common</td>
<td>97</td>
<td>0.249</td>
<td>ALL BFM 95</td>
<td>47</td>
<td>First CR</td>
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<td>6</td>
<td>M</td>
<td>58</td>
<td>Pre-B</td>
<td>97</td>
<td>0.327</td>
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<td>8</td>
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<tr>
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<td>20</td>
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<td>66</td>
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<td>42</td>
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<td>Pro-B</td>
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<td>0.246</td>
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<td>First CR</td>
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<td>92</td>
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<td>ALL BFM 95</td>
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<td>Common</td>
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<td>0.155</td>
<td>ALL BFM 95</td>
<td>30</td>
<td>First CR</td>
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<tr>
<td>23</td>
<td>M</td>
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<td>Common</td>
<td>97</td>
<td>0.312</td>
<td>ALL BFM 95</td>
<td>56</td>
<td>First CR</td>
</tr>
</tbody>
</table>

* pro-B, TdT+, HLA-DR+, CD19+, CD24+, CD10+; common, TdT+, HLA-DR+, CD19+, CD24+, CD10+; pro-B, TdT+, HLA-DR+, CD19+, CD24+, CD10+, CyIgM+.
during the first three courses are in second continuous CR between 17 and 34 months. The remaining patients showed only a gradual decrease in TEL-AML1:β-actin ratio. Most noteworthy, despite the limited observation time at present, subsequent relapses have occurred only in the group of molecular nonresponders (at 9, 23, and 38 months). Thus, the kinetics of TEL-AML1:β-actin decrease during the first few courses of relapse therapy seems to provide invaluable information and measurable evidence enabling clinicians to assign patients more appropriately to therapeutic alternatives at relapse, i.e., chemotherapy or higher-risk transplantation procedures.

No comparison of molecular response to therapy and MRD between initial and relapsed ALL has been reported. The comparatively slower reduction of TEL-AML1:β-actin ratio in relapsed ALL detected in this study might reflect either differences in the time point of BM analysis based on the design of the polychemotherapy regimen or a higher resistance of leukemic cells. Whereas treatment in standard- and medium-risk groups of frontline trial ALL BFM 95 is applied more continuously and molecular evaluation of response during induction is performed at time points without any therapeutic intervals, relapse trial ALL-REZ BFM consists of alternating courses of polychemotherapy in 3-week intervals. Assessment of molecular and morphological remission before the next course might thus influence MRD monitoring by allowing a stronger increase of leukemic cells during hematological recovery and resulting in higher TEL-AML1 transcript levels at similar diagnostic time points.

The expression of the chimeric TEL-AML1 transcript in leukemic cells was normalized by using the β-actin housekeeping gene product as endogenous reference (29, 30). Both TEL-AML1 and β-actin showed similar expression levels in leukemic cells at diagnosis and the same degradation rates within 36–48 h permitting final quantification of TEL-AML1 transcripts in relation to β-actin expression. To ensure optimal conditions for multicentric trials, sampling and RNA processing of leukemic cells should be guaranteed within 36–48 h. The choice of the endogenous control is crucial for standardization and comparison of results generated by different investigators (34, 35).
The kinetics of leukemic cell reduction in childhood ALL by real-time PCR can alternatively be measured on the DNA level (36–38). Clonal TCR and immunoglobulin gene rearrangements constitute unique clone-specific markers and can be detected in nearly every ALL. To its disadvantage, this approach is cost-, time-, and labor-intensive, involving the sequencing of each clonal rearrangement, the individual design of clone-specific primers, and the optimization of each real-time PCR. Furthermore, oligoclonality and clonal evolution of immunoglobulin/TCR rearrangement require the use of at least two DNA markers to avoid false RP-PCR results. Fusion transcript (RNA-) based detection of MRD has the advantage of stability of clonal marker at different stages of disease and of higher sensitivity because of multiple copies per cell but instead has to cope with the disadvantage of RNA stability, the additional step of reverse transcription before PCR, and the fact that recurrent leukemia-specific fusion transcripts are detected only in 30–40% of childhood ALL (5–7).

The presented RP-PCR method is an appropriate tool to quantify TEL-AML1 transcript levels in relation to an endogenous control both for sensitive assessment of response to cytotoxic treatment and for MRD monitoring in 20% of childhood ALL. Individual TEL-AML1 kinetics can be rapidly obtained making this technique most suitable for routine diagnostic procedures and beneficial for clinical decisions. Whether this approach addresses the crucial issue of risk of relapse and early relapse detection correctly is presently handicapped by the long duration of remission inherent to TEL-AML1 ALL requiring longer follow-up times. Generally, all of the clinical studies addressing the prognostic value of detection of MRD should consider the duration of remission when addressing therapeutic decisions concerning reduction, intensification, or prolongation of therapy for this molecular-defined ALL subset.

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